ENHANCED IMMUNOGENICITY USING LEUKOTOXIN CHIMERAS

Description

Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Patent Application Serial No. 07/779,171, filed 16 October 1991, from which priority is claimed pursuant to 35 USC §120 and which is hereby incorporated by reference in its entirety.

Technical Field

The present invention relates generally to immunological carrier systems. More particularly, the invention pertains to leukotoxin-antigen chimeras which demonstrate enhanced immunogenicity as compared to the immunogenicity of the antigen alone.

Background of the Invention

Subunit vaccines are vaccines which are devoid of intact pathogen cells. These vaccines are usually composed of substantially purified antigens. Such vaccines are generally preferable to compositions which use attenuated or inactivated pathogens. However, many subunit vaccines which include proteins, such as peptide hormones and bacterial and viral antigens, require the help of a carrier protein in order to elicit a strong immune response. This is especially true for small proteins or endogenous substances, such as hormones, which are poorly immunogenic.

The carrier serves to non-specifically stimulate T helper cell activity and to direct the antigen to the antigen presenting cell, where the antigen is processed and presented at the cell surface in the context of molecules of the major histocompatibility complex (MHC).

Several carrier systems have been developed for this purpose. For example, small peptide antigens are often coupled to protein carriers such as keyhole limpet haemocyanin (Bittle, J. L., et al., *Nature* (1982) 298:30-33), tetanus toxoid (Muller, G., et al., *Proc. Natl. Acad. Sci. U.S.A.* (1982) 79:569-573), ovalbumin, and sperm whale myoglobin, to produce an immune response. However, carriers may elicit strong immunity not relevant to the peptide antigen and this may inhibit the immune response to the peptide vaccine on secondary immunization (Schutze, M. P., et al, *J. Immun.* (1985) 135:2319-2322).

Antigen delivery systems have also been based on particulate carriers. For example, preformed particles have been used as platforms onto which antigens can be coupled and incorporated. Systems based on proteosomes (Lowell, G. H., et al., *Science* (1988) 240:800-802), immune stimulatory complexes (Morein, B., et al., *Nature* (1984) 308:457-460), and viral particles such as HBsAg (Neurath, A. R., et al., *Mol. Immunol.* (1989) 26:53-62) and rotavirus inner capsid protein (Redmond, M. J., et al., *Mol. Immunol.* (1991) 28:269-278) have been developed.

Other carrier systems have been devised using recombinantly produced chimeric proteins that self assemble into particles. For example, the yeast retrotransposon, Ty, encodes a series of proteins that assemble into virus like particles (Ty-VLPs; Kingsman, S. M., and A. J. Kingsman *Vacc.* (1988) 6:304-306). Foreign genes have been inserted into the *Ty*A gene and expressed

in yeast as a fusion protein. The fusion protein retains the capacity to self assemble into particles of uniform size.

Other chimeric protein particles have been examined such as HBsAg, (Valenzuela, P., et al., *Bio/Technol.* (1985) 3:323-326; U.S. Patent No. 4,722,840; Delpeyroux, F. N., et al., *Science* (1986) 233:472-475), Hepatitis B core antigen (Clarke, B. E., et al., Vaccines 88 (Ed. H. Ginsberg, et al., 1988) pp. 127-131), Poliovirus (Burke, K. L., et al., *Nature* (1988) 332:81-82), and Tobacco Mosaic Virus (Haynes, J. R., et al., *Bio/Technol.* (1986) 4:637-641). However, these carriers are restricted in their usefulness by virtue of the limited size of the active agent which may be inserted into the structural protein without interfering with particle assembly.

Gene fusions provide a convenient method for the production of chimeric proteins. The expression of chimeric proteins affords an efficient means of linking a carrier protein to a desired antigen.

Pasteurella haemolytica produces a cytotoxin which is a leukotoxin. See, e.g. Gentry et al. *Vet. Immunology and Immunopathology* (1985) 9:239-250; Shewen, P.E., and Wilkie, B.N. *Infect. Immun.* (1987) 55:3233-3236. The gene encoding this cytotoxin has been cloned and expressed in bacterial cells. Lo et al. *Infect. Immun.* (1985) 50:667-671; U.S. Patent No. 5,055,400. The leukotoxin has been used as an antigen in vaccine formulations to fight shipping fever pneumonia in livestock (See, e.g. U.S. Patent No. 4,957,739) as well as to produce chimeric molecules for use in vaccines against shipping fever (see, e.g. International Publication No. WO 92/03558, published 5 March 1992; and U.S. Patent No. 5,028,423). However, the use of leukotoxin as a carrier molecule to increase the immune

response of antigens associated therewith has not heretofore been described.

Disclosure of the Invention

The present invention is based on the construction of novel gene fusions between the *P. haemolytica* leukotoxin gene and a nucleotide sequence encoding a selected antigen. These constructs produce a chimeric protein that displays enhanced immunogenicity when compared to the immunologic reaction elicited by administration of the antigen alone.

In one embodiment, the present invention is directed to an immunological carrier system comprising an immunogenic chimeric protein. The chimeric protein comprises a leukotoxin polypeptide fused to a selected antigen, whereby the leukotoxin portion of the chimeric protein acts to increase the immunogenicity of the antigen. In particularly preferred embodiments, the selected antigen is somatostatin (SRIF), gonadotropin releasing hormone (GnRH) or rotavirus viral protein 4 (VP4).

Also disclosed are vaccine compositions comprising the chimeric proteins and a pharmaceutically acceptable vehicle and methods of using the same.

In another embodiment, the subject invention is directed to DNA constructs encoding the chimeric proteins. The DNA constructs comprise a first nucleotide sequence encoding a leukotoxin polypeptide operably linked to a second nucleotide sequence encoding the selected antigen.

In yet another embodiment, the subject invention is directed to expression cassettes comprised of (a) the DNA constructs above and (b) control sequences that direct the transcription of the construct whereby

the constructs can be transcribed and translated in a host cell.

In another embodiment, the invention is directed to host cells transformed with these expression cassettes.

Another embodiment of the invention provides a method of producing a recombinant polypeptide. The method comprises (a) providing a population of host cells described above and (b) growing the population of cells under conditions whereby the polypeptide encoded by the expression cassette is expressed.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts the structure of the leukotoxin gene of *P. haemolytica* cloned in *E. coli* (Plasmid pAA114).

Figure 2 depicts the structure of Plasmid pAA352 wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the β-lactamase gene (ampicillin resistance); ori is the ColE1-based plasmid origin of replication; lktA is the *P. haemolytica* leukotoxin structural gene; and lacI is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figures 3A through 3I (SEQ ID NOS:1 and 2) show ~~Figure 3 shows~~ the nucleotide sequence and predicted amino acid sequence of leukotoxin 352 (LKT 352) from plasmid pAA352. Both the structural gene for LKT 352 and the sequences of the flanking vector regions are shown.

Figure 4 (SEQ ID NOS: 3-8) shows the nucleotide sequences of SRIF, GnRH and bovine rotavirus VP4, used in the construction of the leukotoxin-antigen gene fusions.

Figure 5 shows the structure of Plasmid pAA496 carrying a leukotoxin-SRIF (LKT-SRIF) gene fusion wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the ß-lactamase gene (ampicillin resistance); lktA is the *P. haemolytica* leukotoxin structural gene; SRIF is the somatostatin structural gene; and lac1 is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figure 6A through 6J (SEQ ID NOS: 9 and 10) show ~~Figure 6 depicts~~ the nucleotide sequence and predicted amino acid sequence of the LKT-SRIF chimeric protein from pAA496.

Figure 7 shows the structure of Plasmid pAA502 carrying a leukotoxin-GnRH (LKT-GnRH) gene fusion wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the ß-lactamase gene (ampicillin resistance); lktA is the *P. haemolytica* leukotoxin structural gene; GnRH is the gonadotropin releasing hormone structural gene; and lac1 is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figure 8A through 8J (SEQ ID NOS: 11 and 12) show ~~Figure 8 shows~~ the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pAA502.

Figure 9 depicts the structure of Plasmid pAA501 carrying a leukotoxin-VP4 (LKT-VP4) gene fusion wherein tac is the hybrid trp::lac promoter from *E. coli*; bla represents the ß-lactamase gene (ampicillin resistance); lktA is the *P. haemolytica* leukotoxin structural gene; VP4 is the bovine rotavirus viral

protein 4 (232-255) structural gene; and lacI is the *E. coli* lac operon repressor. The dir ction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale. Figure 10A through 10J (SEQ ID NOS: 13 and 14) show ~~Figure 10 shows~~ the nucleotide sequence and predicted amino acid sequence of the LKT-VP4 chimeric protein from pAA501.

B

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); Maniatis, Fritsch & Sambrook, Molecular Cloning: A Laboratory Manual (1982); DNA Cloning, Vols. I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. 1984); Animal Cell Culture (R.K. Freshney ed. 1986); Immobilized Cells and Enzymes (IRL press, 1986); B. Perbal, A Practical Guide to Molecular Cloning (1984); the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen." An antigen will include one or more epitopes from a protein molecule, such as but not limited to, bacterial and viral proteins, as well as peptide hormones which elicit an immune response. Additionally, an antigen can comprise one or more identical or different immunogenic repeating sequences of a protein. Specifically excluded from the definition for purposes of this application are cytokines such as interleukin-1 (IL1), interleukin-2 (IL2), interleukin-3 (IL3), interleukin-4 (IL4), and gamma-interferon (γIFN).

The term "leukotoxin polypeptide" intends a polypeptide derived from a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (Highlander et al., *DNA* (1989) 8:15-28), where X is Lys, Asp, Val or Asn. (SEQ ID NO:15) Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee, C.A., and Lo, R.Y.C. *Infect. Immun.* (1987) 55:3233-3236; Lo, R.Y.C., *Can. J. Vet. Res.* (1990) 54:S33-S35; Welch, R.A., *Mol. Microbiol.* (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, R.Y.C., *Can. J. Vet. Res.* (1990) 54:S33-S35). In addition, the term "leukotoxin polypeptide" refers to a leukotoxin polypeptide which is chemically synthesized, isolated from an organism expressing the same, or recombinantly produced. Furthermore, the term

intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native leukotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogs. Although native full-length leukotoxins display leukotoxic activity, the term "leukotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of native leukotoxins. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al., *Infect. Immun.* (1985) 50:667-67; Lo et al., *Infect. Immun.* (1987) 55:1987-1996; Strathdee, C.A., and Lo, R.Y.C., *Infect. Immun.* (1987) 55:3233-3236; Highlander et al., *DNA* (1989) 8:15-28; Welch, R.A., *Mol. Microbiol.* (1991) 5:521-528.

By "LKT 352" is meant a protein which is derived from the *lktA* gene present in plasmid pAA352 (Figure 2, ATCC Accession No. 68283). The nucleotide sequence and corresponding amino acid sequence of this gene are described in International Publication No. WO91/15237 and shown in ~~Figure 3~~ Figure 3A through 3I (SEQ ID NOS: 1 and 2). The gene encodes a truncated leukotoxin, having 931 amino acids, which lacks the cytotoxic portion of the molecule. The derived LKT 352 is not necessarily physically derived from the sequence present in plasmid pAA352. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the protein functions to enhance the immunogenicity of the antigen with which it is associated.

A "hapten" is a molecule containing one or mor epitopes that does not stimulate a host's immune system to make a humoral or cellular response unless linked to a carrier.

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site."

An "immunological response" to an antigen or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest.

An "immunogenic protein" or "immunogenic amino acid sequence" is a protein or amino acid sequence, respectively, which elicits an immunological response in a subject to which it is administered.

A leukotoxin-antigen chimera displays "increased immunogenicity" when it possesses a greater capacity to elicit an immune response than the corresponding antigen alone. Such increased immunogenicity can be determined by administering the particular leukotoxin-antigen and antigen controls to animals and comparing antibody titers against the two using standard assays such as radioimmunoassays and ELISAs, well known in the art.

By "carrier system" is meant a system which includes a molecule that serves to increase the immunogenicity of an antigen administered therewith, as defined above. Without being bound by any particular

theory, the molecule may function to increase the immunogenicity of the antigen by presenting the same to cells of the immune system, such as antigen presenting cells, macrophages, follicular dendritic cells, B cells and T cells; or by stimulating the immune system to respond at a level greater than that observed when the antigen is administered alone.

By "subunit antigen composition" is meant a composition containing at least one immunogenic polypeptide, but not all antigens, derived from or homologous to an antigen from a pathogen of interest. Such a composition is substantially free of intact pathogen cells or particles. Generally, a "subunit antigen composition" is prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or recombinant analogs thereof.

The term "protein" is used herein to designate a naturally occurring polypeptide. The term "polypeptide" is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

"Native" proteins or polypeptides refer to proteins or polypeptides recovered from a source occurring in nature. Thus, the term "native leukotoxin" would include naturally occurring leukotoxin and fragments thereof.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "rotavirus VP6 protein" refers to the art-recognized major viral protein of the inner capsid from any species or strain within the family Reoviridae. See, e.g., Kapikian et al., 1985. Examples of rotavirus strains from which the VP6 protein can be isolated and employed in the present invention include, but are not limited to, Simian SA-11, human D rotavirus, bovine UK rotavirus, human Wa or W rotavirus, human DS-1 rotavirus, rhesus rotavirus, the "O" agent, bovine NCDV rotavirus, human S2 rotavirus, human KUN rotavirus, human 390 rotavirus, human P rotavirus, human M rotavirus, human Walk 57/14 rotavirus, human Mo rotavirus, human Ito rotavirus, human Nemoto rotavirus, human YO rotavirus, human McM2 rotavirus, rhesus monkey MMU18006 rotavirus, canine CU-1 rotavirus, feline Taka rotavirus, equine H-2 rotavirus, human St. Thomas No. 3 and No. 4 rotaviruses, human Hosokawa rotavirus, human Hochi rotavirus, porcine SB-2 rotavirus, porcine Gottfried rotavirus, porcine SB-1A rotavirus, porcine OSU rotavirus, equine H-1 rotavirus, chicken Ch.2 rotavirus, turkey Ty.1 rotavirus, bovine C486 rotavirus, and strains derived from them. Thus the present invention encompasses the use of VP6 from any rotavirus strain, whether from subgroup I, subgroup II, or any as yet unidentified subgroup, as well as from any of the serotypes 1-7, as well as any as yet unidentified serotypes. Such VP6 proteins can be used as immunologic carriers of polypeptides. These carrier molecules comprise amino acid sequences of rotavirus VP6 amino acid sequences which are unique to the class, or any member of the class, of VP6 polypeptides. Such unique sequences of VP6 proteins are referred to as a "rotavirus VP6 inner capsid protein amino acid sequence." VP6 carriers are further disclosed in U.S. Patent No. 5,071,651, incorporated herein by reference in its entirety.

A carrier that is "substantially homologous to a rotavirus VP6 inner capsid protein or a functional fragment thereof" is one in which at least about 85%, preferably at least about 90%, and most preferably at least about 95%, of the amino acids match over a defined length of the molecule. A "functional fragment" of a rotavirus VP6 inner capsid protein is a fragment with the capability of acting as a carrier molecule for the novel chimeric proteins of the instant invention.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo* or *in vitro*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vivo* or *in vitro* when placed under the

control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3′ to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3′ terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" another coding sequence when RNA polymeras will transcribe the two coding sequences into mRNA, which is then translated into a chimeric polypeptide encoded by the two coding sequences. The coding sequences need not be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired chimeric protein.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra; DNA Cloning, vols I & II, supra; Nucleic Acid Hybridization, supra.

The term "functionally equivalent" intends that the amino acid sequence of the subject fusion protein is one that will elicit an immunological response, as defined above, equivalent to an unmodified immunogenic leukotoxin-antigen chimeric protein.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

A composition containing A is "substantially free of" B when at least about 85% by weight of the total of A + B in the composition is A. Preferably, A comprises at least about 90% by weight of the total of A

+ B in the composition, more preferably at least about 95%, or even 99% by weight.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms or the disease of interest (therapy).

B. General Methods

Central to the instant invention is the discovery that leukotoxin polypeptides, when coupled to selected antigens, are able to increase the immunogenicity of the antigen as compared to the immunogenicity of the antigen when presented alone. Thus, leukotoxin polypeptides can act as carrier proteins for the presentation of a desired antigen to the immune system. Accordingly, the chimeric proteins can be formulated into vaccine compositions which provide enhanced immunogenicity to the antigen presented therewith. The fusion of the leukotoxin gene to the selected antigen further functions to facilitate purification of the chimeric protein from cells expressing the same.

The leukotoxin carrier is especially useful for the presentation of small or endogenous peptide antigens, including peptide hormones, and bacterial and viral antigens, which typically elicit poor immune responses when presented without the aid of a carrier. Exemplified herein are leukotoxin chimeras which include leukotoxin fused to small peptide hormones -- somatostatin (SRIF) and gonadatropin releasing hormone (GnRH). SRIF-14 has 14 amino acids and GnRH possesses 10 amino acids. The nucleotide sequences of SRIF and GnRH are depicted in Figure 4. (SEQ ID NOS:3-8) Because the sequences are relatively short, they can easily be generated using synthetic techniques, as described further below. Because these hormones are

small in size and are ndogenous to several mammals such as humans, bovines etc., these substances require the use of carrier proteins in order to elicit an adequate immune response in such mammals. Immunization with these hormones can regulate growth rate, lactation and reproductive efficiency. A detailed discussion of SRIF can be found in ~~allowed U.S. Patent Application~~ U.S. Pat. No. 5,210,156 No. ~~07/539,236~~, filed 18 June 1990, which is incorporated herein by reference in its entirety. GnRH is further discussed in U.S. Patent No. 4,975,420, incorporated herein by reference in its entirety.

Also exemplified herein is a chimera comprised of leukotoxin and bovine rotavirus viral protein 4 (VP4). VP4 (molecular weight 86,719), functions as the viral hamagglutinin and forms the spike-like projections protruding from the surface of the virus. Antibodies capable of neutralizing the virus bind to the tip of the spike. VP4 appears to play a major role in viral attachment during infection. The nucleotide sequence of VP4 is depicted in Figure 4. For a further discussion of rotavirus infection and VP4, see, Redmond, M.J. et al. in Viral Diseases (Ed. E. Kurstak, Marcel Dekker, New York, 1991, pp. 387-404); and International Publication No. WO/9207941, published 14 May 1992, both incorporated herein by reference in their entirety. Although the invention is described with respect to these particular proteins, leukotoxin polypeptides, or proteins functionally equivalent and substantially homologous thereto, can be easily fused to other antigens, based on the disclosure herein, in order to increase the immunogenicity thereof.

The leukotoxin-antigen complex can be conveniently produced recombinantly as a chimeric protein. The antigen portion of the chimera can be fused

either 5′ or 3′ to the leukotoxin portion of the molecule.

Actively growing cells of *P. haemolytica* have been shown to secrete leukotoxin which can be cloned, the gene encoding the same isolated, and fused with a gene encoding a desired antigen, using techniques well known in the art. The resulting chimeric proteins can be expressed and used to immunize subjects against the particular antigen fused to leukotoxin.

The nucleotide sequence coding for full-length *P. haemolytica* A1 leukotoxin has been determined. See, e.g., Lo, R.Y.C. *Infect. Immun.* (1987) 55:1987-1996; U.S. Patent No. 5,055,400, incorporated herein by reference in its entirety. *P. haemolytica* leukotoxin can be produced using recombinant techniques and purified from, for example, bacterial cells. The leukotoxin can also be purified from native bacteria using immunoadsorbent chromatography.

Similarly, the coding sequences for numerous antigens are known or can be determined. Again, these antigens can be purified using standard techniques.

Purification of the above proteins, using standard techniques including those described herein, permits the sequencing of the same by any of the various methods known to those skilled in the art. For example, the amino acid sequences can be determined by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art. Furthermore, fragments of the proteins can be tested for biological activity and active epitopes used in compositions in lieu of the entire protein.

Once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be

prepared and used to screen DNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., DNA Cloning: Vol. I, supra; Nucleic Acid Hybridization, supra; Oligonucleotide Synthesis, supra; T. Maniatis et al., supra.

First, a DNA library is prepared. The library can consist of genomic DNA from *P. haemolytica* (for the isolation of the leukotoxin gene) or from appropriate cells or viruses (for the isolation of the desired antigen gene). Once the library is constructed, oligonucleotides to probe the library are prepared and used to isolate the gene encoding the desired protein. The oligonucleotides are synthesized by any appropriate method. The particular nucleotide sequences selected are chosen so as to correspond to the codons encoding a known amino acid sequence from the desired protein. Since the genetic code is degenerate, it will often be necessary to synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long, and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in corresponding nucleic acid sequences, particularly if this lengthy and/or redundant region is highly characteristic of the protein of interest. It may also be desirable to use two probes (or sets of probes), each to different regions of the gene, in a single hybridization experiment.

Automated oligonucleotide synthesis has made the preparation of large families of probes relatively straightforward. While the exact length of the probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

The selected oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin, using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors, such as the length of the probe and whether the probe is derived from the same species as the library, or an evolutionarily close or distant species. The selection of the appropriate conditions is within the skill of the art. See, generally, Nucleic Acid hybridization, supra. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a sufficient degree of nucleic acid homology (e.g., at least about 75%), as opposed to nonspecific binding. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a gene for the desired protein.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for

expression. The complet sequence is assembl d from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al. *Science* (1984) 223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, generally, DNA Cloning: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

Suitable restriction enzymes can then be employed to isolate the appropriate antigen gene or leukotoxin gene and these sequences can be ligated together and cloned to form a leukotoxin-antigen fusion gene.

The fusion gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the chimeric protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding

sequence may or may not contain a signal peptide or leader sequence. The chimeric proteins of the present invention can be expressed using, for example, native *P. haemolytica* promoter, the *E. coli* tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular fusion coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular chimeric protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors

described above. Alternatively, the coding sequence can be cloned dir ctly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogs of the chimeric proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The chimeric protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the

media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform *E. coli* and pooling and screening individual colonies using polyclonal serum or monoclonal antibodies to the desired antigen.

The chimeric proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The proteins of the present invention or their fragments can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of the present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing

cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., Hybridoma Techniques (1980); Hammerling et al., Monoclonal Antibodies and T-cell Hybridomas (1981); Kennett et al., Monoclonal Antibodies (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the antigen of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against.

Animals can be immunized with the compositions of the present invention by administration of the chimeric protein, or an active fragment thereof, or an analog thereof. The chimeric protein can consist of leukotoxin fused to an epitope of the desired antigen, as defined above. Thus, if the fragment or analog of the fusion protein is used, it will include the amino acid sequence of leukotoxin, or a fragment of the same which interacts with the immune system to increase the immunogenicity of the antigen or epitope thereof, linked to the antigen of interest.

Prior to immunization, it may be desirable to further increase the immunogenicity of the particular chimeric protein, or an analog of the protein, or particularly fragments of the protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the antigenic peptide may be administered linked to a carrier, in addition to the leukotoxin carrier. For example, a fragment may be

conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the chimeric proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651, and incorporated herein by reference. Also useful is a fusion product of a viral protein and the subject leukotoxin-antigen immunogen made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the fusion proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides

to proteins or cells are known to those of skill in the art.

The novel chimeric proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel chimeric proteins can be constructed as follows. The DNA encoding the particular leukotoxin-antigen chimeric protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant chimeric protein into the viral genome. The resulting TK- recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

It is also possible to immunize a subject with a protein of the present invention, or an immunogenic fragment thereof, or an analog thereof, which is administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically acceptable and compatible with the ac-

tive ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the subject being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the chimeric protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The chimeric proteins can also be presented using implanted mini-pumps, well known in the art.

Furthermore, the chimeric proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, th polypeptide of interest, or an immunologically active fragment thereof, is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal delivery, are also acceptable. Injectable vaccine formulations will contain an effective amount of the active ingredient in a vehicle, the exact amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. With the present vaccine formulations, approximately 1 μg to 1 mg, more generally 5 μg to 100 μg of antigen per ml of injected solution, should be adequate to raise an immunological response when administered. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the particular antigen or fragment thereof, or analog thereof, in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

<u>Deposits of Strains Useful in Practicing the Invention</u>

A deposit of biologically pure cultures of th following strains was made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

These deposits are provided merely as a convenience to those of skill in the art, and are not an admission that a deposit is required under 35 USC §112. The nucleic acid sequences of these plasmids, as well as the amino sequences of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description herein. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

<u>Strain</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
P. haemolytica serotype 1 B122	February 1, 1989	53863
pAA352 in *E. coli* W1485	March 30, 1990	68283

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T_4 DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

cDNA and genomic libraries were prepared by standard techniques in pUC13 and the bacteriophage lambda gt11, respectively. See DNA CLONING: Vols I and II, supra.

P. haemolytica biotype A, serotype 1 ("A1") strain B122 was isolated from the lung of a calf which died of pneumonic pasteurellosis and was stored at -70°C in defibrinated blood. Routine propagation was carried out on blood agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5% (v/v) horse serum (Gibco Canada Ltd., Burlington, Canada). All cultures were incubated at 37°C.

Example 1

Isolation of P. haemolytica Leukotoxin Gene

To isolate the leukotoxin gene, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See, Lo et al., *Infect. Immun.*, supra; DNA CLONING: Vols. I and II, supra; and T. MANIATIS et al., supra. A genomic library was

constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See, Lo et al., *Infect. Immun.*, supra. To confirm this, smaller fragments were recloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5′ to 3′ direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire leukotoxin gene sequence. The structure of this plasmid is shown in Figure 1.

lktA, a MaeI restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the SmaI site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the ptac-based vector pGH432: lacI digested with SmaI. One, pAA342, consisted of the 5′-AhaIII fragment of the lktA gene while the other, pAA345, contained the entire

MaeI fragment described above. The clone pAA342 expressed a truncat d leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3′ end of the lktA gene (StyI BamHI fragment from pAA345) was ligated to StyI BamHI-digested pAA342, yielding the plasmid pAA352. The structure of pAA352 is shown in Figure 2 and the nucleotide sequence and predicted amino acid sequence of *P. haemolytica* leukotoxin shown in ~~Figure 3~~ Figures 3A through 3I (SEQ ID NOS:1 and 2).

Example 2

Construction of LKT-antigen Fusions

Three representative LKT-antigen fusions were constructed as follows. Oligonucleotides containing sequences from the bovine rotavirus VP4, GnRH and SRIF genes were constructed on a Pharmacia Gene Assembler using standard phosphoramidite chemistry. The sequences of these oligonucleotides are shown in Figure 4. (SEQ ID NOS: 3-8) The oligonucleotides were annealed and ligated into the vector pAA352 (ATCC No. 68283, and described above), which had been digested with the restriction endonuclease BamH1. This vector contains the *P. haemolytica* leukotoxin gene. The ligated DNA was used to transform *E. coli* strain JM105 (in the case of SRIF) or MH3000 (for VP4 and GnRH). Transformants containing the oligonucleotide inserts were identified by restriction endonuclease mapping. Plasmid DNA from the *E. coli* MH3000 strains was then isolated and used to transform the strain JM105. The recombinant plasmids were designated pAA496 (LKT-SRIF, Figure 5), pAA502 (LKT-GnRH, Figure 7), and pAA501 (LKT-VP4, Figure 9). The nucleotide sequences of these three fusions are shown in Figures 6, 8 and 10, respectively.

Example 3

Purification of LKT-antigen Fusions

The recombinant LKT-antigen fusions from Example 2 were purified using the following procedure. For each fusion, five to ten colonies of the transformed *E. coli* strains were inoculated into 10 ml of TB broth supplemented with 100 micrograms/ml of ampicillin and incubated at 37°C for 6 hours on a G10 shaker, 220 rpm. Four ml of this culture was diluted into each of two baffled Fernbach flasks containing 400 ml of TB broth + ampicillin and incubated overnight as described above. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in polypropylene bottles, 500 ml volume, using a Sorvall GS3 rotor. The pellet was resuspended in an equal volume of TB broth containing ampicillin which had been prewarmed to 37°C (i.e., 2 x 400 ml), and the cells were incubated for 2 hours as described above.

3.2 ml of isopropyl-B,D-thiogalactopyranoside (IPTG, Gibco/BRL), 500 mM in water (final concentration = 4 mM), was added to each culture in order to induce synthesis of the recombinant fusion proteins. Cultures were incubated for two hours. Cells were harvested by centrifugation as described above, resuspended in 30 ml of 50 mM Tris-hydrochloride, 25% (w/v) sucrose, pH 8.0, and frozen at -70°C. The frozen cells were thawed at room temperature after 60 minutes at -70°C, and 5 ml of lysozyme (Sigma, 20 mg/ml in 250 mM Tris-HCl, pH 8.0) was added. The mixture was vortexed at high speed for 10 seconds and then placed on ice for 15 minutes. The cells were then added to 500 ml of lysis buffer in a 1000 ml beaker and mixed by stirring with a 2 ml pipette. The beaker containing the lysed cell suspension was placed on ice and sonicated for a total of 2.5 minutes (5-30 second bursts with 1 minute cooling between each) with a Braun sonicator, large probe, set at 100 watts power. Equal

volumes of the solution were placed in Teflon SS34 centrifuge tubes and centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The pellets were resuspended in a total of 100 ml of sterile double distilled water by vortexing at high speed, and the centrifugation step repeated. Supernatants were discarded and the pellets combined in 20 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (Tris-buffered saline) and the suspension frozen overnight at -20°C.

The recombinant suspension was thawed at room temperature and added to 100 ml of 8 M Guanidine HCl (Sigma) in Tris-buffered saline and mixed vigorously. A magnetic stir bar was placed in the bottle and the solubilized sample was mixed at room temperature for 30 minutes. The solution was transferred to a 2000 ml Ehrlenmyer flask and 1200 ml of Tris-buffered saline was quickly added. This mixture was stirred at room temperature for an additional 2 hours. 500 ml aliquots were placed in dialysis bags (Spectrum, 63.7 mm diameter, 6,000-8,000 MW cutoff, #132670, from Fisher scientific) and these were placed in 4,000 ml beakers containing 3,500 ml of Tris-buffered saline + 0.5 M Guanidine HCl. The beakers were placed in a 4°C room on a magnetic stirrer overnight after which dialysis buffer was replaced with Tris-buffered saline + 0.1 M Guanidine HCl and dialysis continued for 12 hours. The buffer was then replaced with Tris-buffered saline + 0.05 M Guanidine HCl and dialysis continued overnight. The buffer was replaced with Tris-buffered saline (no guanidine), and dialysis continued for 12 hours. This was repeated three more times. The final solution was poured into a 2000 ml plastic roller bottle (Corning) and 13 ml of 100 mM PMSF (in ethanol) was added to inhibit protease activity. The solution was stored at -20°C in 100 ml aliquots.

To confirm that the fusion proteins had been isolated, aliquots of each preparation were diluted 20-fold in double distilled water, mixed with an equal volume of SDS-PAGE sample buffer, placed in a boiling water bath for five minutes and run through 12% polyacrylamide gels. Recombinant leukotoxin controls were also run. Western blots of the purification products were performed by reacting the LKT-SRIF preparation with swine anti-SRIF serum at a 1:500 dilution and the LKT-GnRH and LKT-VP4 preparations with mouse anti-VP4 serum at a 1:50 dilution. The only band visible in the LKT-SRIF western blot was that associated with the LKT-SRIF protein sample. No cross-reactivity with the leukotoxin was observed. Both the LKT-GnRH and LKT-VP4 proteins had similar apparent molecular weights, however, the anti-VP4 serum reacted only with the LKT-VP4 fusion protein.

All fusion proteins were expressed at high levels as inclusion bodies. The predicted molecular weights based on the DNA sequences of the three proteins (depicted in Figures 6, 8 and 10) were 101,366 (LKT-SRIF); 100,521 (LKT-GnRH); and 102,120 (LKT-VP4). The molecular weight of the recombinant leukotoxin molecule was 99,338. Both the SRIF and VP4 fusions were shown to react with monospecific antisera against the corresponding peptide.

Example 4

In Vivo Immunologic Activity of LKT-antigen Fusions

To test for enhanced immunogenicity of the LKT-antigen fusions as compared to the antigens alone, LKT-SRIF fusion protein was purified from *E. coli* cultures induced with IPTG, as described in Example 2. Aggregated protein was dissolved by treating with guanidine-HCl at a final concentration of three molar. The leukotoxin

concentration of this material was assayed using a standard quantitative leukotoxin specific ELISA. The assay utilized recombinant leukotoxin in 4 M guanidine-HCl (2 mg/ml) as a standard. Rabbit anti-leukotoxin antiserum was used as a detection and quantitation system.

A vaccine was formulated to a volume of 1 ml by mixing equal volumes of LKT-SRIF, diluted in Hanks Buffered Saline, and Emulsigen Plus (MVP Laboratories, Ralston, Nebraska). Four three month old lambs were immunized twice with 100 micrograms of fusion protein (containing an equivalent of approximately 1.4 micrograms of SRIF peptide). Blood samples were taken 10 days after the second injection and were analyzed for leukotoxin and SRIF specific antisera. All of the animals were found to have anti-leukotoxin titers of greater than 1 in 50,000, as determined by a leukotoxin specific ELISA. SRIF titers were assayed by a radioimmunoassay as described in Laarveld, B., et al., *Can. J. Anim. Sci.* (1986) 66:77-83. Two animals were found to have titers greater than 1 in 100.

To further test the ability of the LKT-SRIF chimeras to induce an anti-SRIF immunological response *in vivo*, and to compare this response to that produced by other SRIF conjugates, the following vaccination trial was performed. Three groups of 8 female pigs, approximately 8 weeks of age (35-50 kg) were used which were Specific Pathogen Free. The animals were maintained in a minimal disease facility and were vaccinated on days 0, 21 and 35 of the trial with the following formulations:

Group 1 -- placebo which was saline formulated in Emulsigen Plus adjuvant containing 15 mg DDA (Kodak) (2 ml);

Group 2 -- LKT-SRIF (250 μg LKT, prepared as described above) formulated in th same adjuvant (2 ml);

Group 3 -- SRIF-avidin, biotinylated SRIF (10 μg) and 2.5 μg avidin, formulated in the same adjuvant (2 ml).

Blood samples were taken on days 0, 21 and 35, allowed to clot, centrifuged at 1500 g, and the serum removed. The serum antibody titers against SRIF were measured using the RIA procedure of Laarveld *et al.*, *Can. J. Anim. Sci.* (1986) 66:77-83.

7 of the 8 animals immunized with the LKT-SRIF formulation produced significant titers against SRIF (>1:700) whereas only 2 of 8 animals immunized with the SRIF-Avidin responded with serum titers of >700.

This example demonstrates that leukotoxin chimeric molecules are highly immunogenic. It has been reported by Laarveld, et al., *Can. J. Animal Sci.* (1986) 66:77, that repeated immunization with greater than 100 micrograms of SRIF peptide conjugated to an ovalbumin carrier was necessary to evoke an immune reaction.

Example 5

In Vivo Immunologic Activity of LKT-GnRH Fusions

To test for the ability of LKT-GnRH to induce an anti GnRH immunological response *in vivo*, and to compare this response to other GnRH carrier conjugates, the following vaccination trial was performed. Three groups of 8 male pigs, approximately 8 weeks of age (35-50 kg) were used which were Specific Pathogen Free. The animals were maintained in a minimal disease facility and were vaccinated on days 0 and 21 of the trial with the following formulations:

Group 1 -- placebo which consisted of saline formulated in Emulsigen Plus adjuvant containing 15 mg of DDA (2 ml);

Group 2 -- LKT-GnRH (250 μg LKT, prepared as described in the previous examples) formulated in the same adjuvant (2 ml);

Group 3 -- VP6-GnRH, 0.5 μg VP6 and 5 μg GnRH, formulated in the same adjuvant (2 ml). The VP6 preparation was made as described in U.S. Patent No. 5,071,651, using the binding peptide described therein.

Blood samples were taken on days 0, 21 and 35, allowed to clot, centrifuged at 1500 g, and the serum removed. The serum antibody titers against GnRH were measured using the RIA procedure of Silversides *et al.*, *J. Reprod. Immunol.* (1985) 7:171-184.

The results of this trial indicated that only those animals immunized with the LKT-GnRH formulation produced significant titers against GnRH (titers >1:70). Neither the placebo nor the VP6-GnRH groups produced anti-GnRH titers. Previously, multiple vaccinations with doses of GnRH of more than 100 μg, conjugated to other carrier proteins, were required to induce anti-hormone titers.

Thus, chimeric proteins including leukotoxin fused to a selected antigen, have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.